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Safety Assessment of Artemether/Lumefantrine/ Tinidazole on the Kidneys of Healthy and Diseased Mice

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Authors' contributions

This work was carried out in collaboration between both authors. Authors EA and UOG designed the study, performed literature search and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Artemether/lumefantrine/tinidazole- (A/L/T) can be use for the treatment of malaria; therefore its safety assessment is imperative. This study assessed its safety on the kidneys of healthy and diseased mice. Fifty four Swiss albino mice were used for this study. Mice were diseased with *Plasmodium berghei* (1×10^7) and treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 4 days. Healthy mice were treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 28 days. At the termination of treatment, the mice were weighed, sacrificed and blood samples were collected and examined for kidney biochemical markers. Kidneys were weighed and evaluated for oxidative stress markers and histology. T, A/L and A/L/T had no significant ($p>0.05$) effects on all evaluated parameters in diseased mice when compared to control. Body weight was decreased whereas kidney weight was increased in healthy mice treated with T, ($p<0.05$), A/L ($p<0.05$) and A/L/T ($p<0.01$) when compared to control. Significantly elevated serum creatinine, urea, uric acid levels with significantly decreased albumin, and total protein levels occurred in healthy mice treated with T

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(p<0.05), A/L (p<0.01) and A/L/T (p<0.001) when compared to control. Altered kidney oxidative stress markers characterized by significantly decreased glutathione, catalase, glutathione peroxidase, superoxide dismutase levels with significantly increased malondialdehyde levels occurred in healthy mice treated with T (p<0.05), A/L (p<0.01) and A/L/T (p<0.001) when compared to control. A/L/T produced tubular necrosis and enlarged Bowman's space in healthy mice. The use of A/L/T as an antimalarial drug may be safe on the kidney, but long term use may cause kidney damage.

Keywords: Artemether-lumefantrine; tinidazole; kidney; toxicity; parasite; mice.

1. INTRODUCTION

The kidney is required for several important functions including the sustenance of homeostasis, detoxification, and excretion of toxic drugs and metabolites, thus it is venerable to toxicant induced damage [1]. A number of drugs including amtimalarial drugs (quinine, and artesunate) have been associated with nephrotoxicity [2]. The presentation of nephrotoxicity varies from an acute or chronic decreased glomerular filtration rate to glomerular and tubular damage [3]. Although renal impairment is often reversible if the offending drug is discontinued, the condition can be costly and may require multiple interventions, including hospitalization [4].

Artemisinin based combinations including artemether/lumefantrine(A/L),artesunate/amodia quine, artesunate/mefloquine are the currently recommended antimalarial therapies [5]. Artemisinin derivatives are recommended worldwide for treatment of malaria because of their high potency, rapid onset of action, broad malaria stage specificity, and favorable safety profile [6]. A/L has shown efficacy against uncomplicated *P. falciparum* malaria and chloroquine resistant *P. falciparum* [7]. It accounted for 73 % of ACTs procured in 2013 [8]. However, A/L may have nephrotoxic effect. Reversible nephrotoxicity, diminished glomerular filtration rate and increased urinary excretion of electrolytes have been reported [9,10].

Tinidazole (5-nitroimidazole drug) (T), which has been proven to be relatively safe is widely used for the treatment of amoebiasis and giardiasis [11]. However, potential antimalaria activity of T has been reported by some scholars. Studies in a chick model parasitized with *Plasmodium gallinaceum* treated with T showed increased survival time [11]. It cures liver stage of relapsing strain of *P. cynomolgi*, in 'Rhesus' macaques (*Macaca mulatta*) and blood stage infection when co-administered with chloroquine [12]. Open label human study showed that T monotherapy followed by weekly doses cleared blood stage infection with no recurrences of *P. vivax* [13]. In previous studies, we showed promising antimalarial activity of T in combination with A/L characterized by decreased paracetamia, prolonged survival time and decreased anemia in a mouse model infected with *P. berghei* [14]. In-view of the potential of animalarial drug combinations to cause nephroroxicity, this study assessed the safety of artemether/lumefantrine/ tinidazole (A/L/T) on the kidneys of healthy and diseased mice.

2. MATERIALS AND METHODS

2.1 Animals and Drugs

Fifty four adult Swiss albino mice (20-25g) of both sexes were supplied by the animal unit of the Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State. The mice were grouped and kept under natural conditions with free access to diet and water. The mice were acclimated for 2 weeks prior to the study. Artemether/lumefantrine (A/L) (IPAC Laboratory, India) and Tinidazole (T) (Norvatis) were used. The doses of T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T used were based on previous antiplasmodial studies [14].

2.2 Parasite Inoculation of Mice and Treatment

CQ sensitive strain of *P. berghei* in donor mice was provided by the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. Thirty Swiss albino mice grouped into 5 (I-V) of n=6 were used. Groups II-V were parasitized i.p with *P. berghei* containing 1×10^7 parasitized erythrocytes and allowed for 3 days. On day 4, the mice were treated as follows: Group 1: (Normal control) and group II (Parasitized control) were orally treated with normal saline (0.2mL). Groups III-V were treated daily with T

(28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 4 days, respectively.

2.3 Treatment of Healthy Mice

In the sub-acute toxicity study, twenty four mice were grouped into four of (n=6) and treated with drugs as follows: Group 1: (Control) was orally treated with normal saline (0.2mL) for 28 days. Groups II-IV were orally treated with T (28.6 mg/kg), A/L (2.3/13.7mg/kg) and A/L/T for 28 days, respectively.

2.4 Animal Sacrifice

After, treatment, the mice were weighed; fasted over night and anesthetized (diethylether). Blood samples were obtained from the heart, centrifuged (1200 rmp for 20minutes) and sera extracted and assessed for biochemical markers. Mice were dissected, kidneys collected and rinsed in saline. Rinsed kidneys were homogenized in buffered (pH 7.4), 0.1 M Tris-HCl solution and centrifuged (2000 rmp for 20 minutes). The homogenates were decanted and assessed for oxidative stress markers.

2.5 Assessments of Serum Biochemical Markers

Sera were estimated for uric acid, creatinine, urea, total protein, albumin, sodium, potassium and bicarbonate using test kits

2.6 Oxidative Stress Marker Assay

Kidney glutathione (GSH) was assayed according to Sedlak and Lindsay [15]. Catalase (CAT) was assayed as explained by Aebi, [16]. Glutathione peroxidase (GPx) was measured according to Rotruck et al. [17]. Superoxide dismutase (SOD) was estimated as reported by Sun and Zigman [18]. Malondialdehyde (MDA) was measured as described by Buege and Aust [19].

2.7 Histology of the Kidney

Dissected kidney tissues were cut and fixed in Bouin's solution for 24hr. Kidney tissues were dehydrated in alcohol-graded series processed and embedded in paraffin wax. Sections (3µm each) were cut and stained with Haematoxylin and Eosin on slides. The slides were examined using a light microscope and relevant sections photographed.

2.8 Statistical Analysis

Data were presented as mean ± SEM and assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test (Graph Pad Prism 5 Software, San Diego, CA USA). Differences are statistically significant at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

3. RESULTS

3.1 Effects of Artemether /Lumefantrine /Tinidazole on Body and Kidney Weights and Serum Biochemical Markers of Parasitized Mice

Treatment with T, A/L and A/L/T for 4 days did not produce significant ($p > 0.05$) effects on body and kidney weights of parasitized mice when compared to normal control (Table 1). Normal ($p > 0.05$) serum electrolytes, creatinine, urea, uric acid, total protein and albumin levels were observed in parasitized mice treated with T, A/L and A/L/T for 4 days when compared to normal control (Table 2).

Table 1. Effects of artemether/lumefantrine/tinidazole on body and kidney weights of healthy and parasitized mice

Treatment	Final body weight (g)		Absolute kidney weight (g)		Relative kidney weight (%)	
	Healthy mice	Parasitized mice	Healthy mice	Parasitized mice	Healthy mice	Parasitized mice
Control	28.73±2.43	27.25±3.77	0.17±0.03	0.16±0.01	0.59±0.02	0.59±0.06
T	23.52±2.90	27.94±2.58	0.20±0.01	0.15±0.04	0.85±0.06*	0.54±0.01
A/L	24.01±2.57	29.61±3.32	0.21±0.05	0.17±0.06	0.87±0.08*	0.57±0.09
A/L/T	20.99±3.56*	29.96±2.11	0.25±0.09*	0.16±0.02	1.12±0.05 ᵀᵀ	0.54±0.04

*T: Tinidazole, A/L: Artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole ,Data as mean ± SEM, n=6, * p<0.05 in comparison to control (Healthy mice), ᵀᵀ p<0.01 in comparison to control (Healthy mice), SEM: Standard error of mean*

Table 2. Effect of artemether/lumefantrine/tinidazole on serum kidney biochemical markers of healthy and parasitized mice

Treatment	Healthy mice					
	Creatinine (mg/dL)	Urea(mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	Parasitized mice Urea (mg/dL)	Uric acid (mg/dL)
Control	0.50±0.03	7.27±0.04	1.37±0.13	0.56±0.09	7.41±0.07	1.42±0.24
T	0.97±0.07*	10.91±0.09*	2.89±0.09*	0.54±0.03	7.33±0.01	1.40±0.45
A/L	1.36±0.03**	13.83±0.36**	4.05±0.06**	0.57±0.07	7.30±0.05	1.38±0.67
A/L/T	2.67±0.08π	20.14±2.71π	7.76±0.72π	0.53±0.01	7.40±0.08	1.35±0.44

*T: Tinidazole, A/L: Artemether/lumefantrine, A/L/T: Artemether/lumefantrine/tinidazole, Data as mean ± SEM, n=6, * p<0.05, ** p<0.01, π p<0.001 when compared to control (Healthy mice), SEM: Standard error of mean*

Table 3. Effect of artemether/lumefantrine/tinidazole serum electrolytes of healthy and parasitized mice

Treatment	Healthy mice				Parasitized mice			
	K (mmol/L)	Na (mmol/L)	Cl (mmol/L)	HCO3 (mmol/L)	K (mmol/L)	Na (mmol/L)	Cl (mmol/L)	HCO3 (mmol/L)
Control	4.33±0.03	120.25±11.7	133.74±12.6	12.14±1.71	4.12±0.06	110.16±10.6	127.30±9.22	13.31±1.65
T	4.30±0.90	117.02±10.5	130.42±10.7	12.37±01.42	4.10±0.01	109.17±12.1	125.56±10.6	13.65±1.14
A/L	4.27±0.57	115.64±12.3	128.36±10.5	12.74±1.63	4.09±0.19	107.34±10.9	123.45±11.8	13.36±1.90
A/L/T	4.25±0.56	114.91±10.1	126.23±11.9	12.60±1.24	4.06±0.24	105.45±11.4	122.37±12.5	13.73±1.40

T: Tinidazole, A/L: Artemether/lumefantrine, AL/T: Artemether/lumefantrine/tinidazole, Data as Mean ± SEM, n=6, SEM: Standard error of mean

Table 4. Effect of artemether/lumefantrine/tinidazole on kidney oxidative stress markers of healthy mice

Treatment	MDA nmole/mg protein	GSH µmole/mg protein	CAT U/mg protein	SOD U/mg protein	GPx U/mg protein
Control	0.12 ± 0.02	8.44 ± 0.73	25.64 ± 2.01	14.85± 2.51	15.34 ± 1.00
T	0.25 ± 0.04*	6. 05± 0.87*	20.80 ± 2.33*	11.01 ± 2.33*	11.61 ± 0.32*
A/L	0.46 ± 0.06**	4.35± 0.45**	15.36 ± 1.71**	9.00 ± 0.41**	8.14 ± 0.71**
A/L/T	0.70 ± 0.03 ¶	2.22± 0.27 ¶	10.82 ± 0.79 ¶	6.01 ± 2.70 ¶	5.40 ± 0.26 ¶

T: Tinidazole, A/L: Artemether/lumefantrine, AL/T: Artemether/lumefantrine/tinidazole, MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, Data as Mean ± SEM, n=6, p<0.05, **p<0.01, ¶ p<0.001 compared to control, SEM: Standard error of mean*

3.2 Effects of Artemether/Lumefantrine/ Tinidazole on Body and Kidney Weights and Serum Biochemical Markers of Healthy Mice

Body weight was significantly decreased whereas kidney weight was significantly increased in healthy mice treated with T ($p<0.05$), A/L ($p<0.05$) and A/L/T ($p<0.01$) for 28 days when compared to control (Table 1). Serum creatnine, urea and uric acid levels were significantly increased whereas total protein and albumin levels were significantly decreased in healthy mice treated with T ($p<0.05$), A/L ($p<0.01$) and A/L/T ($p<0.001$) for 28 days when compared to control (Table 3). Serum electrolytes (Sodium, potassium, chloride, and bicarbonate) were normal ($p>0.05$) in healthy mice treated with T, A/L and A/L/T for 28 days when compared to control (Table 3).

3.3 Effects of Artemether /Lumefantrine/ Tinidazole on Kidney Oxidative Stress Markers and Histology of Healthy Mice

Kidney antioxidants (SOD, GSH, CAT and GPx) were significantly decreased in healthy mice treated with T ($p<0.05$), A/L ($p<0.01$) and A/L/T ($p<0.001$) for 28 days when compared to control (Table 4). On the other hand, MDA levels were significantly increased in healthy mice treated with T ($p<0.05$), A/L ($p<0.01$) and A/L/T ($p<0.001$) for 28 days when compared to control (Table 4). Kidney of control mice showed normal histology (Fig. A). Kidney of healthy mice treated with A/L showed enlarged Bowman's space and tubular necrosis (Fig. B). Kidney of healthy mice treated with T showed tubular necrosis (Fig. C). Kidney of healthy mice treated with A/L/T showed enlarged Bowman's space and tubular necrosis (Fig. D).

4. DISCUSSION

Previous study has shown the antiplasmodial activity of A/L/T in a mouse model infected with *P. bergei* [14]. Thus study further assessed the safety of A/L/T on the kidneys of healthy and *P. bergei* infected mice. Perturbations in body and organ weights are used in experimental studies to ascertain the toxic effects of chemical substances [20]. In this study, body and kidney weights were normal in parasitized mice treated with A/L/T. However, body weight was decreased whereas kidney weight was increased in healthy mice treated with A/L/T. Biomarkers of renal function are used to estimate the severity and nature of kidney injury, and to decipher appropriate therapy. Biomarkers including serum creatinine, urea and uric acid, total protein and albumin can be used to assess the extent of kidney perturbation. Creatinine gives a clear picture of glomerular filtration rate whereas total protein can accurately detect early kidney perturbation as well as chronic kidney perturbation [21]. In this study, renal function markers were normal in parasitized mice treated with A/L/T whereas, sub-acute toxicity study in healthy mice showed impaired renal function markers caused by A/L/T. This was characterized by elevated serum creatinine, urea and uric acid levels with decreased serum total protein and albumin levels. Electrolytes are positively and negatively charged ions, which are found within cells and extracellular fluids, including intestinal fluid, blood, and plasma. Electrolytes play important functions in cellular function, intermediary metabolism, enzyme activities and electrical gradients [22]. Fluctuations in serum electrolytes can predict the functional status of the kidney [23]. The current study observed normal serum electrolytes in parasitized mice and healthy mice treated with A/L/T. Oxidative stress is a state in which oxidation exceeds the

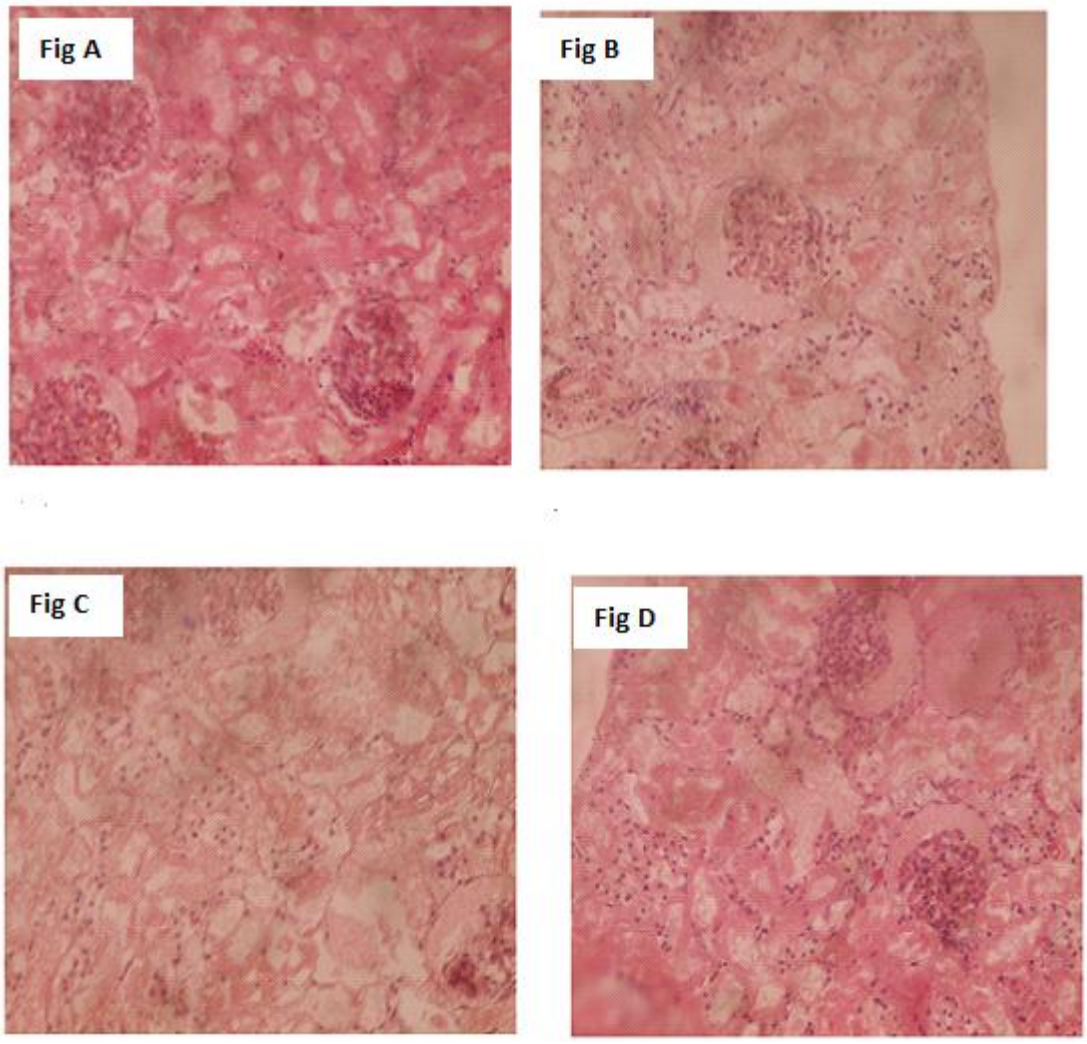

Fig. A. Kidney of control mice showed normal histology, Fig. B. Kidney of healthy mice treated with A/L showed enlarged Bowman's space and tubular necrosis, Fig. C. Kidney of healthy mice treated with T showed tubular necrosis, Fig. D. Kidney of healthy mice treated with A/L/T showed enlarged Bowman's space and tubular necrosis

antioxidant systems, which can cause hazardous damage to biomolecules. It can also alter physiologic adaptation phenomena and the regulation of intracellular signal transduction. Low levels of antioxidants have been experimentally used to assess oxidative stress [24]. Lipid peroxidation is a chain reaction by which unsaturated fatty acids (cell membrane components) are oxidized in various pathological conditions. Many markers of lipid peroxidation have been proposed, including lipid peroxides, malondialdehyde, and 4-hydroxynonenal [24]. In the current study, healthy mice treated with A/L/T showed decreased kidney antioxidants with increased kidney malondialdehyde levels. This observation connotes oxidative stress. The kidneys of A/L/T treated healthy mice were distorted as marked by tubular necrosis and enlarged Bowman's space. This observation correlates with changes in serum renal function markers and kidney oxidative stress markers observed in A/L/T treated rats. A/L has been shown to cause nephrotoxicity characterized by alterations in serum creatinine urea and uric acid levels [25] which is consistent with the observation in this study. Also, changes in kidney oxidative stress markers and histology in A/L treated mice have been previously reported [25]. Studies have shown that T is relatively safe [11], but this study observed signs of nephrotoxicity in

treated healthy mice characterized by altered kidney histology, and serum renal function indices. Treatment with T also produced alterations in kidney oxidative stress markers of healthy mice.

5. CONCLUSION

The use of A/L/T as an antimalrial drug may be safe on the kidney, but long term use may cause kidney damage considering the dose and route used for this study. Cautioned is advised with long term use.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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